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1. ABSTRACT

Current theories suggest that atherosclerotic and restenotic lesions result from imbalances between systems that are proinflammatory/fibroproliferative versus the endogenous inhibitory systems that normally limit inflammation and vascular wound repair. Abnormalities in one of the major regulatory pathways, the transforming growth factor- β (TGF- β) system, has been characterized in both animal models and in human lesions and lesion-derived cells. TGF- β signaling is capable of regulating many of the key aspects of atherosclerosis and restenosis: inflammation, chemotaxis, fibrosis, proliferation, and apoptosis. There are significant decreases in TGF- β activity in patients with atherosclerosis, and equally important changes in the way cells respond to TGF- β during atherogenesis. Evidence from multiple sources indicates that experimental modulation of TGF- β activity, or TGF- β responses, changes the course of atherosclerosis and intimal hyperplasia. Cells derived from human lesions produce adequate TGF- β levels, but are resistant to the antiproliferative and apoptotic effects of TGF- β . An evolving theory describes TGF- β as a major orchestrator of the vascular repair process, with observable defects in its production, activation, and cellular responses during the atherosclerotic and restenotic processes.

2. INTRODUCTION

2.1. Atherosclerosis

Cardiovascular diseases remain the largest cause of death and morbidity in the Western hemisphere. Despite major advances in diagnosis, risk management, and treatment, roughly 40% of all deaths are attributable to heart disease, stroke, and atherosclerosis (1). Americans have about a 2-fold greater risk of dying of atherosclerotic diseases than cancer. The major pathophysiological causes of vascular disease are raised 'fibro-fatty' lesions which occlude blood flow and trigger thrombosis, as shown histologically in Figure 1. Atherosclerotic vascular changes are a major contributing factor to life-threatening events such as myocardial infarction, stroke, aneurysm, and pulmonary embolism. The incidence of coronary and aortic atherosclerosis increases strongly with advancing age, such that by the age of 65 as much as 50% of the coronary artery surface can be covered by raised atherosclerotic lesions (2).

2.2. Angioplasty/Restenosis

Patients presenting with clinical and angiographic evidence of occlusive coronary atherosclerosis are usually offered either coronary bypass surgery or angioplasty. Balloon angioplasty, typically with

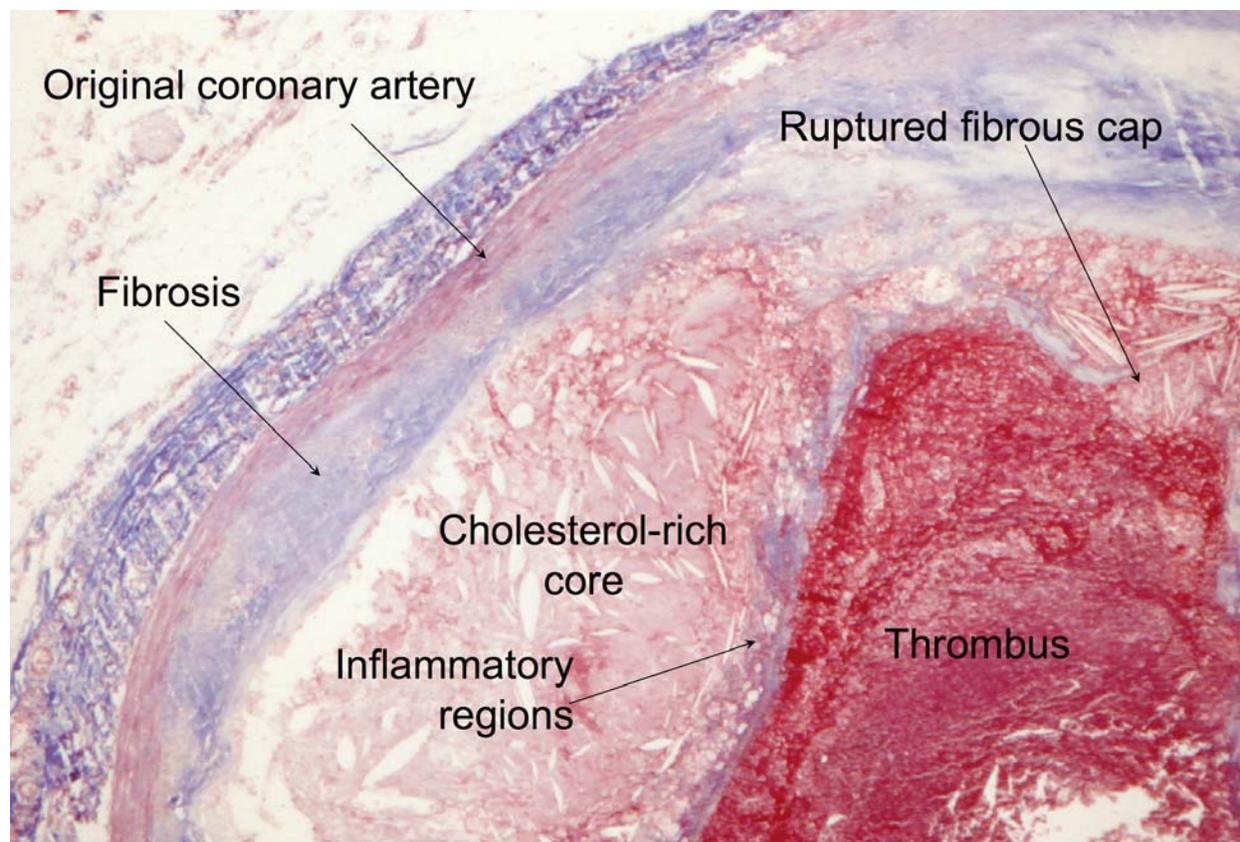


Figure 1. Histological features of atherosclerosis in humans. A Masson's trichrome stained, coronal section of the left anterior descending artery of a thrombotically occluded atherosclerotic lesion which led to a fatal myocardial infarction. Blue stain indicates collagen-rich regions, pale red indicates normal cellular regions, and deep red indicates the mural thrombus in the lumen of the artery.

endovascular stent placement, is performed on more than 450,000 patients per year in the U.S., and achieves initial reperfusion in more than 95% of cases. Given the high success rate, short hospitalization, and minimally invasive nature, angioplasty is likely to remain a treatment of choice for coronary and peripheral atherosclerosis. The initially high success rate of angioplasty is offset by progressive fibroproliferative reclosure of the artery, termed restenosis, over the 3 to 6 month period post-angioplasty, though this complication is significantly reduced by newer drug-eluting stents.

2.3. Pathophysiology of atherosclerosis and restenosis

Atherosclerosis, derived from the Greek 'athero' for gruel, and 'skleros' for hard, is thought to result from chronic or repetitive insults to the vessel wall which trigger a hyperplastic response with prominent inflammatory components. Many of the damaging agents are known: elevated lipids, especially in their oxidized/modified forms, hydrocarbons from smoking, elevated glucose from diabetes, elevated blood pressure and shear forces due to hypertension, and potentially, autoimmune or infectious damage. While these vascular insults are tolerated and repaired efficiently in younger people, advancing age is associated with imbalances in the repair processes leading

to accumulation of repair cells, excess extracellular matrix, inefficient removal of cholesterol-rich debris, and persistent inflammation. As shown in Figure 1, these lesions become lethal when the plaque ruptures, exposing the cholesterol and procoagulant-rich debris to blood, precipitating thrombosis and occluding blood flow to the heart, brain, or other vital organs.

The restenotic process may be an interesting microcosm of the atherosclerotic process because it is a highly-defined, acute vascular insult. Balloon angioplasty causes severe arterial injury, creating intimal "flaps" of partially dissected lesion (Figure 2), and fissures in the lesion, with accompanying mural thrombus. Histologically, these flaps and fissures are consumed with a fibroproliferative lesion over a period of several months. The course of injury-induced intimal hyperplasia probably involves: 1) endothelial injury and connective layer dissections, 2) mural thrombosis, 3) migration of smooth muscle cells (SMC) and myofibroblasts, and infiltration of circulating monocytes/lymphocytes, 4) increased cell proliferation within the neointima, 5) accumulation of extracellular matrix, 6) reendothelialization, 7) partial or complete resolution via apoptosis (3) and 8) tissue remodeling involving wound contraction. As shown in

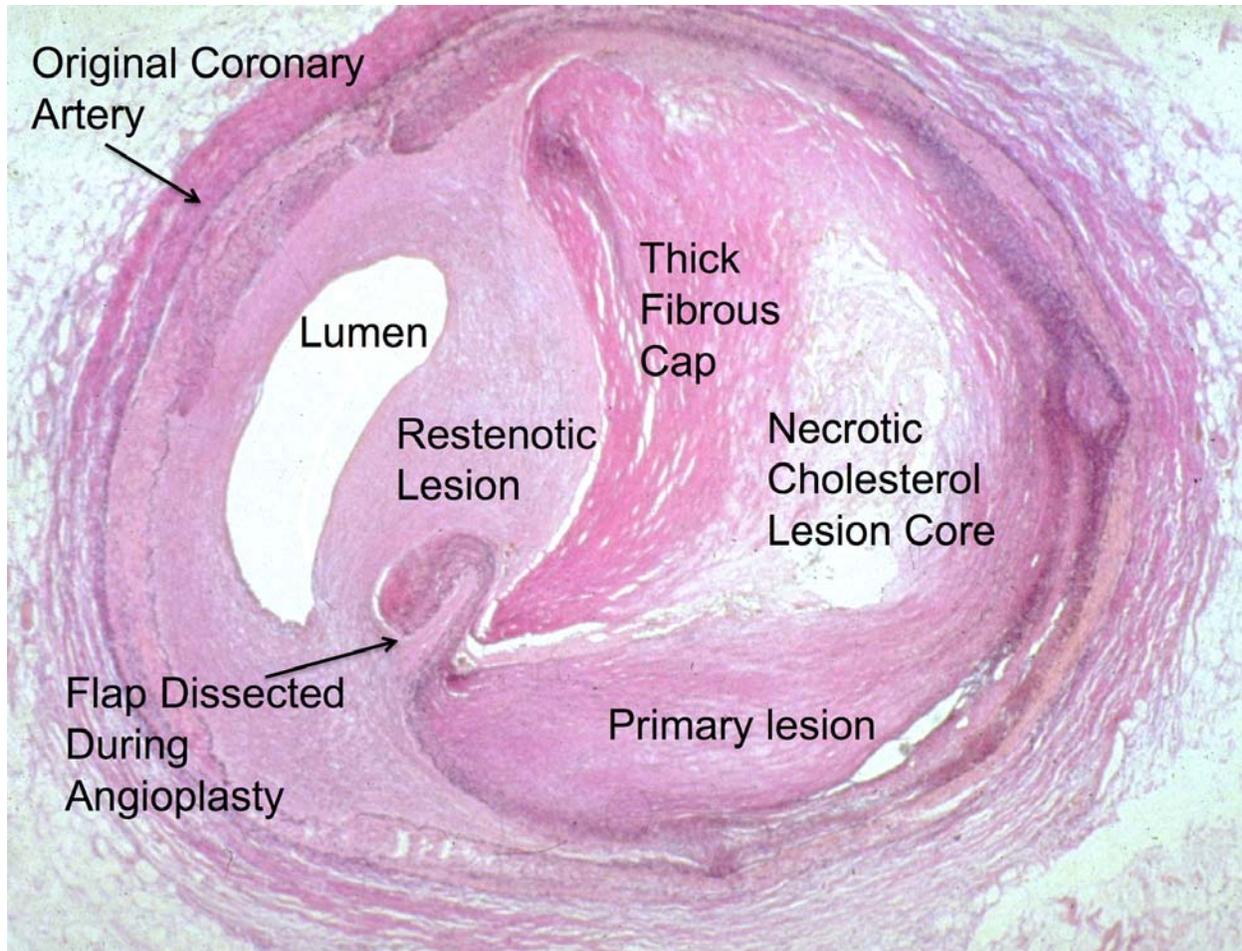


Figure 2. Restenosis of a human coronary artery lesion revascularized by balloon angioplasty. A hematoxylin/eosin-stained coronal section through a diseased human left coronary descending artery which had been revascularized by balloon angioplasty 3 months prior to death from unrelated causes. The major histological features are marked to indicate the original, primary atherosclerotic lesion which occluded almost 90% of the artery (right), prior to balloon inflation which temporarily restored 50% lumen diameter. During angioplasty, a portion of the original lesion dissected, creating an intimal ‘flap’. Progressive, concentric fibroproliferative expansion of a restenotic lesion consumed all but 10% remaining lumen diameter.

Figure 2, these lesion are principally fibrotic and can largely reclose an artery in the absence of drug-eluting stents.

2.4. Regression

Rats and rabbits both develop vascular lesions after balloon injury, though these lesions will spontaneously regress over a period of weeks after injury. However, injury to old rats leads to a more aggressive proliferative wave, and a failure to resolve the lesion after reendothelialization. This excessive response in the aged artery is due to intrinsic changes in the artery wall (4-6). Likewise, evidence from the PDAY study, and others, strongly indicates that atherosclerotic lesions occur reversibly in young adults, but lesions become more persistent with advancing age (7). Serial angiographic analysis in humans indicates that restenosis occurs in most patients after angioplasty, but the majority of patients show spontaneous regression of the lesion (8). Thus, one of the

major questions is why some patients heal normally, without persistent hyperplasia, while others develop an occlusive vascular hyperplasia in response to the same injury. A reasonable theory is that atherosclerosis and restenosis reflect an underlying failure in the *regression* of wound repair due to a defect in the apoptotic process (9).

3. PRODUCTION AND LOCALIZATION OF TGF- β 1 DURING THE REPAIR OF VASCULAR DAMAGE

Essentially all cells present in the arterial wall during vascular damage and repair are capable of producing transforming growth factor- β (TGF- β). TGF- β is a small family of TGF- β 1, β 2, and β 3 in mammals, with an extended family of TGF- β -like proteins including the bone morphogenic proteins (BMPs), inhibins, activins, and growth/differentiation factors (GDFs). Immunostaining reveals high levels of active TGF- β 1 within fibroproliferative regions of the human lesion (Figure 3).

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TGF- β 1 is produced as a 26 kD dimer which then reassociates with a precursor sequence to form a latent complex (10). Latent TGF- β can be activated by acidic conditions (11), plasmin (12), transglutaminase (13), and thrombospondin (14). TGF- β and its latent complex binds to several key extracellular matrix proteins that include fibronectin (15), thrombospondin (16), and type IV collagen (17), which localize TGF- β in the extracellular matrix in its latent form (18). Thus, latent TGF- β is a 'cryptocrine' factor, which resides in a cryptic, latent form that can be activated by local proteolytic activity (19).

TGF- β 1 is a heparin-binding protein (20, 21), and its association with heparin prevents the binding of TGF- β 1 to the activated form of α_2 -macroglobulin, the principal soluble inactivating pathway for TGF- β (21). This action of heparin on TGF- β probably contributes to the antiproliferative effect of heparin on SMC (21, 22). At sites of vascular injury, TGF- β 1 would be released from degranulating platelets in the mural thrombus, and equally importantly, TGF- β 1 production is increased in restenotic lesions (23) and in the balloon injured rat carotid artery (24, 25).

4. EFFECTS OF TGF- β S ON VASCULAR CELLS

TGF- β exerts potent and diverse actions on each of the cell types involved in vascular disease (26). TGF- β frequently exerts *bifunctional* effects that are dependent upon the *context* in which the particular cell type encounters the TGF- β signal. Biologically, this context-dependent effect would allow TGF- β to orchestrate an entire repair process that might involve opposite effects at different times. For additional perspectives on the relationship of TGF- β to vascular disease, other excellent recent reviews are highly recommended (27).

4.1. Endothelial cells

Endothelial cells tend to be strongly inhibited by TGF- β both with respect to their proliferation and migration (28, 29). This inhibition is associated with increases in extracellular matrix and proteoglycan synthesis (30). The *in vitro* antiproliferative effect of TGF- β may explain *in vivo* studies of TGF- β treatment which have observed delays in reendothelialization of the denuded artery (31). It seems likely that the anti-migratory and anti-proliferative effects of TGF- β are most relevant to the anti-angiogenic effect of TGF- β . Importantly, TGF- β can have a dose-dependent bifunctional effect on angiogenesis induced by VEGF and FGF (32) which parallels its ability to stimulate endothelial migration and proliferation at low concentrations, but inhibit both at higher concentrations (33). Conceptually, this *in vitro* dose-dependent effect probably mimics an *in vivo* concentration gradient of TGF- β designed to attract cells from a distance, but then retain them at the site of injury for repair.

4.2. Smooth muscle cells (SMC)

Smooth muscle cells (SMC), and closely related myofibroblasts, which compose much of the atherosclerotic lesion, have been extensively studied for their response to TGF- β . Typically, TGF- β is a potent inhibitor of migration

and proliferation of SMC (34, 35), though in rat aortic SMC, TGF- β can stimulate the production and release of mitogens, such as PDGF, which can have mitogenic effects on SMC (36), particularly when the SMC are quiescent or highly confluent. SMC derived from the chick embryonic cardiac neural crest are of ectodermal origin and are thought to compose the proximal portions of the coronary arteries. These ectodermal cells are growth-stimulated by TGF- β , while cells derived from the mesoderm are growth inhibited (37). TGF- β is a particularly potent modulator of the human SMC phenotype, as it regulates cytoskeletal actin reorganization and cell spreading (35).

The effects of TGF- β on apoptosis are cell-type dependent (38) and context-dependent. Typically, TGF- β will induce apoptosis of both vascular SMC and endothelial cells. However, in circumstances where apoptosis is initiated by other factors, TGF- β can exert a protective effect. Cells derived from human vascular lesions appear prone to spontaneous apoptosis (39), though once the culture is established the lesion-derived cells are typically completely resistant to TGF- β -induced apoptosis (40). Physiologically, TGF- β -induced apoptosis may be a critical feature of wound or lesion regression after repair is completed.

4.3. Macrophages

Macrophages exhibit a complex interaction with TGF- β because the effect is dependent upon their state of activation. TGF- β tends to suppress the activation of monocytes to macrophages (41), a key step in atherogenesis. TGF- β suppresses both the proliferation and migration of cells in the monocyte/macrophage lineage. However, once activated, TGF- β stimulates macrophages to produce urokinase, leading to plasmin generation (42). Within the lesion, macrophages commonly colocalize to regions containing active TGF- β (40, 43).

4.4. Lymphocytes

Lymphocytes are potently suppressed by TGF- β under most conditions tested. TGF- β induces apoptosis in B and T lymphocytes in culture at very low concentrations (44). Within the vascular lesion, lymphocytes express the Type I but not the Type II receptor, suggesting that limited resistance to TGF- β may be required for their function in the plaque (40).

5. EFFECTS OF TGF- β AND TGF- β NEUTRALIZATION ON VASCULAR DISEASE

Overexpression of TGF- β , via direct cDNA transfection into the artery wall at the time of vascular injury, markedly increases intimal and medial thickness, principally by increasing the extracellular matrix component (45). Likewise, infusion of TGF- β at the time of balloon catheter injury in the rat also causes a two-fold increase in the thickness of the neointima and increases the content of matrix in the lesion (24). Rabbit arteries showed a similar fibrotic response to vascular injury with infusion of TGF- β (46).

Likewise, infusions of a neutralizing antibody to TGF- β at the time of balloon catheter injury to the rat aorta

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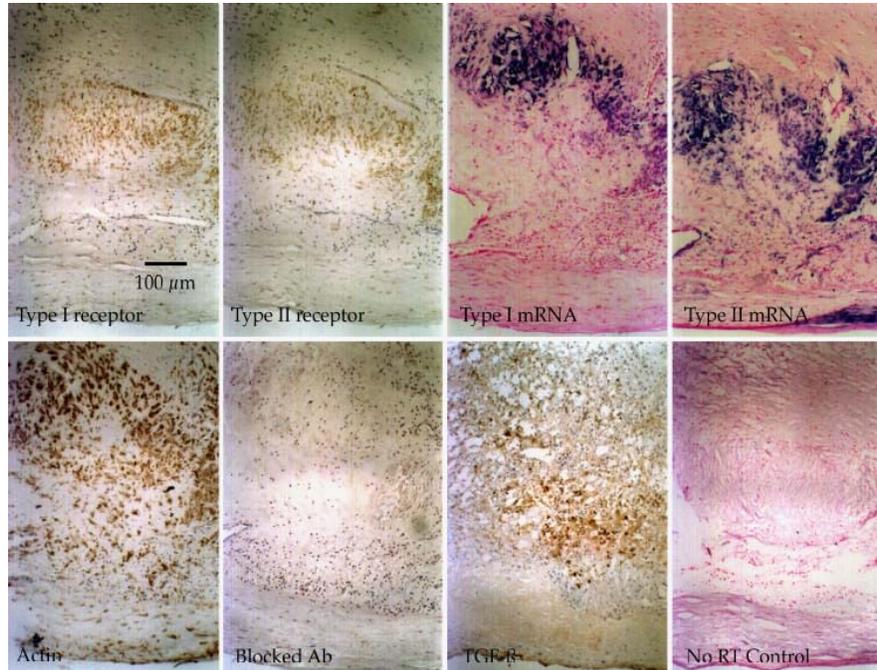


Figure 3. Expression of TGF- β 1, and Type I and Type II receptors in human atherosclerotic lesions. Human lesions were acquired from patients undergoing surgical endarterectomy of the carotid artery, which were formaldehyde-fixed, paraffin-embedded, and immuno-stained with mono-specific polyclonal antibodies to the TGF- β Type I receptor (upper left) or the Type II receptor (upper left central). Sections were lightly counterstained with hematoxylin (light blue). Both antibodies identify the same cell group (light brown) at the base of this lesion (E12). The scale bar (100 μ m) also delimits the junction of the lesion, above, from the tunica media, below. Pre-incubation of the antibodies with an excess of peptide antigen dramatically reduced immunoreactivity (Blocked Ab, Type II, lower left central). Serial sections were immunostained with a monoclonal antibody to the contractile α -actin isoform characteristic of vascular smooth muscle and myofibroblasts (Actin, lower left). TGF- β 1 was identified with a monoclonal antibody specific for the active protein (lower right central). Adjacent sections were analyzed for the expression of the Type I and Type II receptor mRNAs using RT-PCR/ISH to produce a blue color reaction in positive cells, counterstained with Nuclear Fast Red (upper right panels). Identical reactions which omitted the reverse transcriptase step (no RT) did not show positive reactions (lower right). Reprinted from (40).

markedly reduced intimal hyperplasia and fibrosis (25). Experimentally defeating the TGF- β response in T cells by expression of dominant negative Type II receptor expression in ApoE (47) and LDL receptor (48) knockout mice, or by systemic treatment with a soluble TGF- β Type II receptor (49), all cause exaggerated atherosclerosis. However, systemic treatment by injection of a soluble Type II receptor into ApoE $-/-$ mice reduced lesion size and switched it from a fibrotic to an inflammatory lesion (50).

5.1. Expression of TGF- β s and receptors during vascular repair

5.1.1. Animal models

Circulating levels of TGF- β may be an important factor that modulates the vessel wall response to injury. Apo(a), which interferes with the normal activation of TGF- β 1 in the artery wall, contributes to excessive cell proliferation in animal models of lipid-induced injury (51). After vascular injury to the rat carotid, expression of TGF- β 1 mRNA in the vessel wall is elevated within 6 hours, peaks at 24 hours, with sustained elevations for up to 2 weeks, coinciding with the development of the fibroproliferative neointima (24). TGF- β 1 production is increased in arteries after mechanical injury (24, 25), hypercholesterolemia (43), and DOC/salt hypertension

(52). Both Type I and Type II receptors for TGF- β 1 are increased after balloon injury to the rabbit (46).

5.1.2. Human atherosclerosis

Immunohistochemical analysis of early human lesions indicates that TGF- β 1 and TGF- β 3 isoforms are present in the lesion, in association with SMC, macrophages, and foam cells. Both the Type I Alk5 receptor and the Type II receptor were detected at elevated levels in the early fibrofatty streak (53). However, as shown in Figure 3, advanced atherosclerotic lesions show only isolated areas that express either active TGF- β 1 or the receptors (40, 53). The majority of the advanced fibrous lesion expresses low and variable levels of the Type I receptor, but generally much lower levels of the Type II receptor.

5.2. Circulating/soluble levels of TGF- β in atherosclerosis

TGF- β has important autocrine, paracrine, and endocrine effects, and thus, it is important to consider soluble and circulating levels. Accumulating evidence suggests that TGF- β levels in plasma are reduced in patients with atherosclerosis (54-56). Vessel wall levels, much of which would be interstitial and matrix-associated, are also reduced in atherosclerotic regions, further

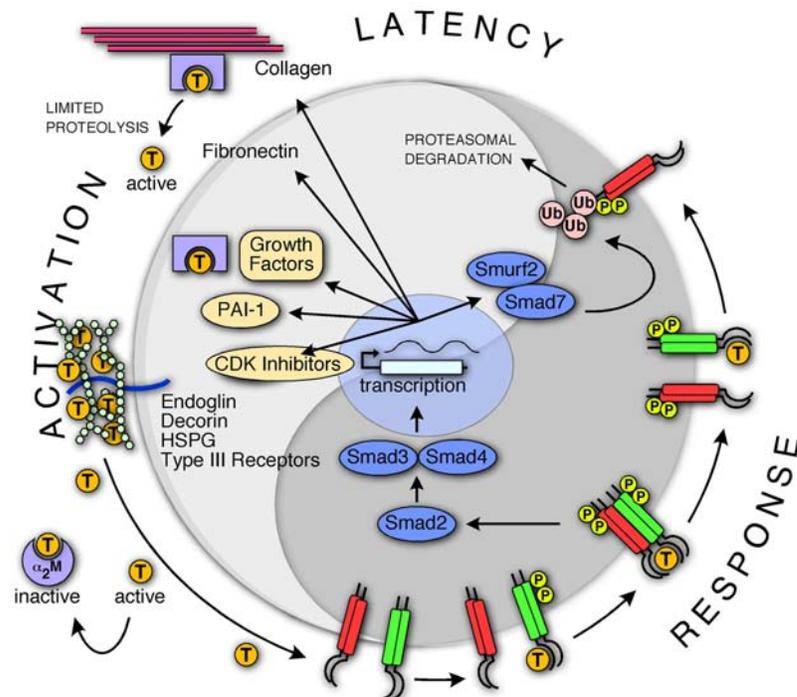


Figure 4. Schematic flowchart of TGF- β signal control in the vessel wall. The TGF- β signal is defined both by multi-step control of the TGF- β growth factor, and by the receptors and signaling systems on the responsive cells. TGF- β has multiple levels of intrinsic latency which embed the active protein into extracellular matrix in a ‘cryptocrine’ state, awaiting activation by proteolysis or other matrix injury. Upon activation, TGF- β is rapidly inactivated by α_2 -macroglobulin (α_2 M), if it is not stabilized by heparan sulfate-like proteoglycans including endoglin, decorin and biglycan. If the active protein contacts a cell expressing TGF- β receptors, then a signaling cascade involving receptor heteromultimerization, SMAD phosphorylation and dimerization, and transcription is initiated. Negative feedback factors are among the transcriptionally activated signals, including SMAD7, Smurf2, and related factors that can cause proteasome-mediated degradation of TGF- β receptors and signaling SMADs.

suggesting a reduced bioactive TGF- β signal during atherogenesis (27). The combination of reduced levels, and reduced responsiveness would greatly reduce the overall TGF- β signal in the atherosclerotic environment. On the basis of this and other evidence, a “protective cytokine” theory of atherosclerosis has been forwarded, which highlights the potential importance of reduced TGF- β bioactivity in atherosclerosis (57).

5.3. Resistance to the antiproliferative effect of TGF- β 1

The sharp increase in atherosclerotic disease with age implies that underlying age-related changes in the vascular wall may exacerbate vascular injury (4, 5), partially due to age-related resistance to inhibitors such as TGF- β (6, 58, 59). SMC derived from old animals produce normal amounts of both active and latent TGF- β 1 (60). However, the old SMC showed no inhibition of DNA synthesis in response to TGF- β 1 over the range 0-5 ng/ml, while young SMC were inhibited 50% at 50 pg/ml (60). A similar age-dependent response to TGF- β has been observed in SMC derived from the spontaneously hypertensive rat (61). Interestingly, elevated cholesterol levels reduce the responsiveness to TGF- β in vascular cells by modulating Type II/Type receptor binding profiles (62, 63).

In both rat and human cells, this acquired resistance is associated with a preferential down-regulation of the Type II receptors (40, 60), although additional changes in negative

regulators of signaling, such as Smurf2, have been observed (64). Transfection of Type II receptors partially corrects the response of human LDC and old rat SMC (65), suggesting that the intracellular signaling system remains at least partially functional. The remaining resistance could be due to changes in the key SMAD signaling pathways that have been observed in cells of the fibrofatty lesion (66). A small subset of patients possess lesion cells with acquired mutations in a microsatellite region of the Type II receptor (67, 68). However, it is likely that regulation of Type II receptor levels, and changes in the intracellular signaling pathways are the major modes of resistance. For instance, changes in the E3 ubiquitin ligases, Smurf1 and Smurf2, could account for reduced TGF- β signaling via SMAD interactions (69, 70). Recently, a number of other potentially important TGF- β signaling modulators have been described, including Arkadia (71), Nedd4-2 (72), and WWP1 (73). Also, TGF- β is capable signaling both through SMAD-dependent, and SMAD-independent pathways to modulate vessel wall functions (74).

6. SUMMARY AND CONCLUSIONS

The evidence is compelling that TGF- β activity and responses are key modulators of the normal, and abnormal vascular repair processes. As diagrammed schematically in Figure 4, the TGF- β system involves tight regulation of this signal at multiple levels. Reduced TGF- β

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activity is a feature of atherosclerosis, both in the vessel wall and in circulating levels, thereby favoring a pro-inflammatory environment. Concurrent with the decreased TGF- β activity, there is a reduced and altered response to TGF- β by subsets of cells in the atherosclerotic lesion, which may be symptomatic of broader changes because lesion cells are also resistant to the effects of glucocorticoids (75). TGF- β resistance, on one hand, may allow vascular repair cells to tolerate hostile environments and repair the damage, but if it persists, lesion cells would excessively repair the artery wall. Thus, determining the molecular basis of the imbalances in this key system may be critical in understanding occlusive vascular diseases. While it is tempting to categorize TGF- β as either a 'protective' or 'pathologic' factor, it is more likely that TGF- β is instrumental in both normal physiologic vascular repair, and a component of dysregulated vascular disease.

7. ACKNOWLEDGEMENTS

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